1652

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
BOODHOO et al.	Group Art Unit: 1652
Application No.: 09/996,620	Examiner: Not Yet Assigned
Filed: November 27, 2001	
For: HIGHLY PURIFIED MOCARHAGIN, A COBRA VENOM PROTEASE, POLYNUCLEOTIDES ENCODING SAME AND RELATED PROTEASES, AND THERAPEUTIC USES THEREOF	

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Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

REQUEST FOR CORRECTED PATENT APPLICATION PUBLICATION UNDER 37 C.F.R. § 1.221(b)

On September 12, 2002, the U.S. Patent and Trademark Office published this application as Publication No. US 2002/0127691-A1. The published application contains a number of mistakes that are the fault of the Office and are, in Applicants' view, material. For the Office's convenience, attached hereto are (1) copies of the relevant pages of the originally filed application and (2) marked-up copies of the corresponding pages of the published application containing the mistakes.

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A mistake is material when it affects the public's ability to appreciate the technical disclosure of the patent application publication or determine the scope of the provisional rights that Applicants may seek to enforce upon issuance of a patent. *See* 37 C.F.R. § 1.221(b). The mistakes listed below may affect the public's ability to appreciate the technical disclosure of the patent application publication or to determine the scope of provisional rights.

The mistakes, which are indicated in red ink on the relevant pages of the marked-up copy of the published application attached hereto are listed below with their corrections.

1. At paragraph 0134 on page 5, the Office erroneously added a comma after a semicolon. This mistake is material as it is unclear what the structure of the sentence is in light of this additional punctuation mark, and may affect the understanding of the paragraph.
2. At paragraph 0166 on page 6, the Office converted a tilde symbol (~) to a hyphen (-) where the applications states "the novel ~50k band." This error is material as the tilde symbol is a mathematical operation symbol which means approximately to one of ordinary skill in the art; the hyphen does not have this meaning, but instead, refers to a negative number.

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3. At paragraph 0202 on page 9, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "Purification of Mocarhagin" is meant to be a heading, not part of the process described in Example 1.
4. At paragraph 0206 on page 9, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "Neutrophil/HL 60 Binding Inhibition Assay" is meant to be a heading, not part of the process described in Example 2.
5. At paragraph 0209 on page 10, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "PGSL-1 Digestion Assay" is meant to be a heading, not part of the process described in Example 3.
6. At paragraph 0210 on page 10, the Office erroneously converted "1 mg/mL BSA for 4 h at 4C" to "1 mg/ML BSA for 4 h at 4C." This error is material, as mL is a standard volume measurement (milliliter), whereas ML is unclear because M is used by one of ordinary skill in the art to designate Molarity.

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7. At paragraph 0211 on page 10, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "Peptide Cleavage Assay" is meant to be a heading, not part of the process described in Example 4.

8. At paragraph 0214 on page 10, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "Cloning of Polynucleotide Encoding Mocarhagin Protein" is meant to be a heading only, not part of the process described in Example 5.

9. At paragraph 0216 on page 10, in the second to last line, the Office changed "50 μg/ml" to "50 ug/ml." This error is material, as μ is a prefix meaning one millionth or 10^{-6}. *See Handbook of Chemistry and Physics, 63rd Edition*, F-103 (1983).

10. At paragraph 0218 on page 11, the paragraph numbering has been erroneously added, and should be omitted in the published application. This error is material as "Enterokinase Cleavage of NMM-9ek" is meant to be a heading, not part of the process described in Example 6.

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11. At paragraph 0219, first line of the second column on page 11, the Office erroneously changed "^{35}S" to "35S." This error is material as the superscript number 35 designates the atomic number for this isotope.

12. At paragraph 0220 on page 11, the Office converted a tilde symbol (~) to a hyphen (-) where the applications states "a novel ~50k band." This error is material as the tilde symbol is a mathematical operation symbol which means approximately; the hyphen does not have this meaning, but instead, refers to a negative number.

Applicants request that the Office correct the above-identified mistakes in the published application, which were the fault of the Office. Further, Applicants request that the Office forward to Applicants a copy of the corrected published application or at least a notification of the occurrence or predicted occurrence of the corrected publication once it has been corrected.

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Attorney Docket No. 08702.0001-03000

Applicants believe that no Petition or fee is due in connection with this Request; however, if any Petition or fee is due, please grant the Petition and charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: November 12, 2002

By: Rebecca McNeill
Rebecca McNeill
Reg. No. 43,796

Enclosures:

Copies of the relevant pages of the originally filed application; marked-up copies of corresponding pages of the published application.

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(f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14;

(g) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14 from amino acid 24 to amino acid 592;

(h) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:14 from amino acid 192 to amino acid 592;

(i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:14 encoding a protein having mocarhagin activity;

(j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

(k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

(l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:16;

(b) the amino acid sequence of SEQ ID NO:16 from amino acid 62 to amino acid 462;

(c) fragments of the amino acid sequence of SEQ ID NO:16 encoding a protein having mocarhagin activity; and

(d) the amino acid sequence encoded by the cDNA insert of clone NMM-3 deposited under accession number ATCC 209587;

the protein being substantially free from other mammalian proteins.

Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15;

Fig. 1 depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the size exclusion column as described herein. Multiple protein species of similar molecular weight can be seen in these fractions.

Fig. 2 depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the Mono-S column as described herein. This gel demonstrates the high degree of purity of the mocarhagin material purified as described in Example 1.

Fig. 3 is an SDS-PAGE gel analysis of fractions containing enterokinase-cleaved mocarhagin protein produced by expression of the NMM-9ek construct described below. The dot indicates the novel ~50k band produced by enterokinase cleavage.

Detailed Description of the Invention and Preferred Embodiments

The present invention provides a highly specific metalloproteinase, mocarhagin, which has been purified from the venom of the Mozambiquan spitting cobra, *Naja mossambica mossambica* (a.k.a., *Naja mocambique mocambique*). Mocarhagin cleaves a ten amino acid peptide from the mature N-terminus of PSGL-1 and abolishes the ability of PSGL-1 to bind P-selectin. These results are in accord with the negative charge/sulfated tyrosine cluster at the N-terminus of PSGL-1 being an important determinant of P-selectin recognition in addition to the recognition of carbohydrate structure.

Mocarhagin can be purified from cobra venom according to the method described in the examples below. Other methods of purifying mocarhagin from cobra venom will also be apparent to those skilled in the art. The progress of any purification scheme for mocarhagin can be monitored on the basis of the biochemical characteristics of mocarhagin described herein and the assays for PSGL-1 digestion and neutrophil/HL60 cell binding described below.

A cDNA encoding a mocarhagin protein ("clone NMM-1") has also been cloned from a cobra venom gland library as described in Example 5 below. The nucleotide sequence of the NMM-1 cDNA is reported as SEQ ID NO:5. Clone NMM-1 was deposited with the American Type Culture Collection on January 16,

Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the mocarhagin protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the mocarhagin protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The mocarhagin protein thus purified is substantially free of other mammalian or other host cell proteins and is defined in accordance with the present invention as "isolated mocarhagin protein".

Examples

The following examples are presented to illustrate, not to limit, the present invention.

Example 1

Purification of Mocarhagin

20 grams of crude protein from snake venom (*Naja mossambica mossambica*, Sigma, product no. V1627) were dissolved in 500 mL deionized H_2O and centrifuged at 10 K rpm for thirty minutes at 4 C. The supernatant was loaded

onto a 200 mL Heparin - 650 M affinity column (Toyopearl, Tosohaas) equilibrated with 50mm Tris-HCl ph 7.6 (buffer A) and 0.2M NaCl. The column was first washed extensively (to baseline) and mocarhagin was eluted with a gradient of 0.2 - 1.0 M NaCl in buffer A. Fractions containing the protease as monitored by SDS-PAGE (band with molecular weight ~55 kD) were pooled, concentrated using bentriprep-10 (Amicon) and applied to 21.5 mm ID x 60 CM size exclusion column (G 3000SW, Tosohaas) in PBS at RT. Fractions eluted from the size exclusion column were analyzed by SDS-PAGE (Fig. 1), which showed the presence of multiple proteins of similar molecular weight.

Fractions containing mocarhagin were pooled and applied onto a Mono S 10/10 column (Pharmacia) equilibrated in 50mm HEPES ph 8.0 (buffer B) at RT a O-IM NaCl in buffer B, gradient was used to elute the protein. The fractions were assayed by SDS-PAGE, pooled and frozen at 80 C. The recovery was 2-3 mg of mocarahagin per gram snake venom processed with a purity greater than 95%. Fig. 2 depicts a gel demonstrating the purity of the mocarhagin produced as herein described.

The N-terminal sequence was determined for the process described above as

TNTPEQDRYLQAKKYIEFYVVVDNVMYRKYTGKLHVITXXVYEMNALN (SEQ ID NO:2). The residues indicated in caps (TNTPEQDRYLQAKKYIEFYVVVDNVMYRKY, SEQ ID NO:1) were determined to a higher degree of certainty.

Example 2

Neutrophil/HL60 Binding Inhibition Assay

Neutrophils were isolated from venous blood anticoagulated with heparin (20 units/mL, final concentration) according to the method of Bignold and Ferrante ((1987) J. Immunol. Meth. 96, 29). The neutrophils were >95% pure as evaluated by flow cytometry and >98% viable by trypan blue exclusion. HL60 cells were cultured in RPMI medium supplemented with 10% fetal calf serum. Immediately before use, cells were washed twice with phosphate-buffered saline (0.01 M

sodium phosphate, 0.15 M sodium chloride, pH 7.4). Neutrophils and cultured cells were finally resuspended at 2 x 10^7/mL in RPMI medium supplemented with 1% fetal calf serum. Binding of ^{125}I-labeled P-selectin (Skinner et al.) to neutrophils or HL60 cells was evaluated by incubating ^{125}I-labeled P-selectin (0.5 µg/mL, final concentration) with cells (1 x 10^7/mL, final concentration) at 22°C in a final volume of 200 µl. After 30 min, duplicate 50 µl aliquots were withdrawn and loaded onto 200 µl of 17% (w/v) sucrose in RPMI medium containing 1% fetal calf serum. Neutrophils were pelleted at 8,750 x g for 2 min. After careful aspiration of the supernatant, radiolabel associated with the cell pellets was measured in a -counter. Nonspecific binding of ^{125}I-labeled P-selectin was assessed using a 50-fold excess of unlabeled P-selectin (Skinner et al.).

To examine the effect of pretreatment of neutrophils or HL60 cells with mocarhagin on P-selectin binding, washed cells (2 x 10^7/mL) in RPMI made 1% in fetal calf serum were incubated in the presence or absence of 10 mM EDTA followed by mocarhagin (0.025-100 µg/mL, final concentrations) for 30 min at 22C. P-selectin binding was then either directly assessed or was assessed after centrifugation of the cells, which were then washed twice and finally resuspended in RPMI with 1% fetal calf serum. In some experiments, DFP-treated mocarhagin was employed in place of mocarhagin. To evaluate the effect of supernatant from mocarhagin treated cells on P-selectin binding, HL60 cells at 10^8/mL in 0.01 M Tris, 0.015 M sodium chloride, 0.001 M calcium chloride, pH 7.4, were incubated with mocarhagin (12 µg/mL) for 10 min at 22°C. The supernatant collected following centrifugation at 1000 x g for 10 min was made 0.1% in BSA and loaded onto a heparin Sepharose CL-6B column (0.5 x 5 cm) to remove mocarhagin. The flow through was then tested for its effect on P-selectin binding to HL60 cells.

Example 3

PSGL-1 Digestion Assay

COS cells were cotransfected with three plasmids encoding soluble PSGL-1 (pED.sPSGL-1.T7; Sako et al.), alpha 1,3/1,4 fucosyltransferase (pEA.3/4FT) and soluble PACE (pEA-PACE SOL; Wasley et al. (1993) J. Biol. Chem. 268, 8458-

8465). [^{35}S]Methionine-labeled COS conditioned medium containing sPSGL-1.T7 was digested with 5 µg/mL mocarhagin in TBS, 2 mM calcium chloride; 1 mg/mL BSA for 20 min at 37C. The ability of sPSGL-1.T7 to bind P-selectin was assessed by precipitation with the P-selectin IgG chimera LECγ1 (Sako et al.) preabsorbed onto protein A Sepharose beads in TBS, 2 mM calcium chloride, 1 mg/mL BSA for 4 h at 4C. A control experiment was also performed where the LECγ1 protein A Sepharose beads were pre-treated with mocarhagin and then exhaustively washed prior to presentation of sPSGL-1.T7. For immunoprecipitation analysis of untreated and mocarhagin treated sPSGL-1.T7, the protease was inactivated by the addition of 5 mM EDTA. sPSGL-1.T7 was then immunoprecipitated with anti-PSGL-1 polyclonal antibodies Rb3026 (raised against COS produced sPSGL-1.T7; Sako et al.) or Rb3443 (raised against the N-terminal peptide of PACE cleaved PSGL-1:QATEYEYLDYDFLPE, SEQ ID NO:4).

Example 4

Peptide Cleavage Assay

A digestion buffer (10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, pH 7.5) and a peptide substrate solution (pyroEATEYEYLDYDFLPE (SEQ ID NO:3), 10 mM in DMSO) were prepared. 2.5 µL peptide substrate solution (250 µM final substrate concentration) was combined with mocarhagin sample material (10 µg/mL final mocarhagin concentration) and adjusted to 100 µL with digestion buffer using no less than 75 µL. This mixture was digested at 37C for 16 hours in parallel with a control sample (no mocarhagin added).

50 µL aliquots of the digested samples were run on an RP-HPLC column (Vydac C18 218TP54, 4.6 x 250 mm), using the following solvents: solvent A, 0.1% TFA in H_2O; solvent B, 0.075% TFA in 90% AcN; flow rate 1 mL/min. The presence of peptides in the eluate was measured by absorbance at 214 nm, 260 nm and 280 nm. A positive assay result was indicated by observing elution of two peptide peaks in the tested sample which both elute earlier than the single peptide peak observed in the negative control.

Example 5

Cloning of Polynucleotide Encoding Mocarhagin Protein

Venom glands from five Mozambiquan spitting cobras, *Naja mossambica mossambica*, were dissected at two hour intervals, two to twelve hours following stimulation of venom production. Poly A + RNA was isolated from total RNA of the pooled gland tissue using an Oligotex Direct mRNA kit (Qiagen, Chatsworth, CA). Synthesis of cDNA was performed using Superscript Choice System (Gibco BRL, Gaithersberg, MD) using oligo dT and random hexamer primers, EcoRI adapters. The cDNA was ligated with EcoRI digested lambda Zap II cloning vector (Stratagene, La Jolla, CA).

Using the above cDNA preparation as template, a PCR reaction was performed using degenerate oligonucleotides based on the N-terminal 30 residue amino acid sequence described above. The sequences of the forward primer consisted of 5'- ACNCCNGARCARGAY (SEQ ID NO:19). The sequences of the reverse primer consisted of 5'- RTAYTTYCKRTACAT (SEQ ID NO:20). A resulting 84 bp product was subsequently identified and DNA sequencing confirmed the sequence encoded 30 amino acid residues having a high degree of homology to the previously determined amino acid sequence. Two oligonucleotides 24 nucleotides in length, 5'- CAGGACAGGTACTTGCAGGCCAAA (SEQ ID NO:21) and 5'- ATCGAGTTTTACGTGGTTGTGGAC (SEQ ID NO:22), were synthesized based on the PCR product sequence and used as ^{32}P hybridization probes to screen approximately 10^6 plaques of plated lambda Zap II library. Duplicate sets of Duralose filters (Stratagene, La Jolla, CA) were hybridized seperately with each ^{32}P hybridization probe in 5xSSC, 5x Denhardt's, 0.1% SDS, 50ug/ml yeast tRNA 16hrs @40C. Filters were washed with 4x SSC, 0.1% SDS @ room temperature, then twice at 45C for 30min. Autoradiography was -70C overnight with intensifying screen. Plaques showing positive hybridization to both probes were isolated and ultimately characterized by nucleotide sequencing.

Clones NMM-1, NMM-2, NMM-3, NMM-9, NMM-10, NMM-12 and NMM-13, described above, were isolated by this technique.

Example 6

Enterokinase Cleavage of NMM-9ek

COS cells were transfected with plasmid pED.NMM9ek, which included the cDNA sequence of SEQ ID NO:17 as an insert. This construct contains a novel enterokinase cleavage site between the propeptide and mature peptide of mocarhagin. After 48hours, the transfected cells were washed in serum free medium, labelled with ^{35}S methionine for 6 hours, and the serum free conditioned mediuma was harvested. Purified bovine enterokinase (La Vallie et al., 1993, J. BIOL. Chem. 268:23311-23317) was added at various concentrations to 100ul conditioned medium with 10mM Tris pH8 and 1mM $CaCl_2$, and incubate at 37C overnight. Soy Trypsin Inhibitor resin was added to remove the enterokinase from the reaction mixture. The resin was pelleted by centrifugation and the supernatant was then immunoprecipitated with rabbit polyclonal antibodies raised against mocarhagin purified from cobra venom.

Following SDS-PAGE and autoradiography, a novel ~50kD band appeared in the smple lane where 50 nanograms of purified bovine enterokinase had been incubated with the conditioned medium (see Fig. 3). This band is consistent with the expected molecular weight of the mature protease when the propeptide (~23 kD) is cleaved off.

[0126] (i) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:14 encoding a protein having mocarhagin activity;

[0127] (j) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(h) above;

[0128] (k) a polynucleotide which encodes a species homologue of the protein of (f), (g) or (h) above; and

[0129] (l) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(h) above.

[0130] The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

[0131] (a) the amino acid sequence of SEQ ID NO:16;

[0132] (b) the amino acid sequence of SEQ ID NO:16 from amino acid 62 to amino acid 462;

[0133] (c) fragments of the amino acid sequence of SEQ ID NO:16 encoding a protein having mocarhagin activity; and

[0134] (d) the amino acid sequence encoded by the cDNA insert of clone NMM-3 deposited under accession number ATCC 209587; the protein being substantially free from other mammalian proteins.

[0135] Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

[0136] (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15;

[0137] (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15 from nucleotide 3 to nucleotide 1388;

[0138] (c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:15 from nucleotide 186 to nucleotide 1388;

[0139] (d) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-3 deposited under accession number ATCC 209587;

[0140] (e) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:16;

[0141] (f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:16 from amino acid 62 to amino acid 462;

[0142] (g) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:16 encoding a protein having mocarhagin activity;

[0143] (h) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(g) above;

[0144] (i) a polynucleotide which encodes a species homologue of the protein of (e) or (f) above; and

[0145] (j) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(g) above.

[0146] The present invention also provides a composition comprising a mocarhagin protein, wherein said protein comprises an amino acid sequence selected from the group consisting of:

[0147] (a) the amino acid sequence of SEQ ID NO:18;

[0148] (b) the amino acid sequence of SEQ ID NO:18 from amino acid 197 to amino acid 621;

[0149] (c) fragments of the amino acid sequence of SEQ ID NO:18 encoding a protein having mocarhagin activity; and

[0150] (d) the amino acid sequence encoded by the cDNA insert of clone NMM-9ek deposited under accession number ATCC 209583;

[0151] the protein being substantially free from other mammalian proteins.

[0152] Yet other embodiments provide for a composition comprising an isolated polynucleotide selected from the group consisting of:

[0153] (a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17;

[0154] (b) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17 from nucleotide 67 to nucleotide 1929;

[0155] (c) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:17 from nucleotide 655 to nucleotide 1929;

[0156] (d) a polynucleotide encoding the mature protein encoded by the cDNA insert of clone NMM-9ek deposited under accession number ATCC 209583;

[0157] (e) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:18;

[0158] (f) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:18 from amino acid 197 to amino acid 621;

[0159] (g) a polynucleotide encoding a protein comprising a fragment of the amino acid sequence of SEQ ID NO:18 encoding a protein having mocarhagin activity;

[0160] (h) a polynucleotide which is an allelic variant of a polynucleotide of (a)-(g) above;

[0161] (i) a polynucleotide which encodes a species homologue of the protein of (e) or (f) above; and

[0162] (j) a polynucleotide which hybridizes under stringent conditions to a polynucleotide of (a)-(g) above.

[0163] Compositions comprising an antibody which specifically reacts with the mocarhagin proteins or a fragments thereof having mocarhagin proteolytic activity are also provided.

BRIEF DESCRIPTION OF THE FIGURE

[0164] **FIG. 1** depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the size exclusion column as described herein. Multiple protein species of similar molecular weight can be seen in these fractions.

[0165] **FIG. 2** depicts an SDS-PAGE gel analysis of fractions containing mocarhagin eluted from the Mono-S column as described herein. This gel demonstrates the high degree of purity of the mocarhagin material purified as described in Example 1.

[0166] **FIG. 3** is an SDS-PAGE gel analysis of fractions containing enterokinase-cleaved mocarhagin protein produced by expression of the NMM-9ek construct described below. The dot indicates the novel ~50k band produced by enterokinase cleavage.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

[0167] The present invention provides a highly specific metalloproteinase, mocarhagin, which has been purified from the venom of the Mozambiquan spitting cobra, Naja mossambica mossambica (a.k.a., Naja mocambique mocambique). Mocarhagin cleaves a ten amino acid peptide from the mature N-terminus of PSGL-1 and abolishes the ability of PSGL-1 to bind P-selectin. These results are in accord with the negative charge/sulfated tyrosine cluster at the N-terminus of PSGL-1 being an important determinant of P-selectin recognition in addition to the recognition of carbohydrate structure.

[0168] Mocarhagin can be purified from cobra venom according to the method described in the examples below. Other methods of purifying mocarhagin from cobra venom will also be apparent to those skilled in the art. The progress of any purification scheme for mocarhagin can be monitored on the basis of the biochemical characteristics of mocarhagin described herein and the assays for PSGL-1 digestion and neutrophil/HL60 cell binding described below.

[0169] A cDNA encoding a mocarhagin protein ("clone NMM-1") has also been cloned from a cobra venom gland library as described in Example 5 below. The nucleotide sequence of the NMM-1 cDNA is reported as SEQ ID NO:5. Clone NMM-1 was deposited with the American Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209588. The protein sequence encoded by clone NMM-1 is reported as SEQ ID NO:6. Amino acids 1-23 of SEQ ID NO:6 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

[0170] Four additional full-length cDNAs encoding closely related proteases (clones "NMM-2", "NMM-9", "NMM-12" and "NMM-13") were also isolated from the cobra venom gland library as described in Example 5 below. Each of the proteins encoded by such cDNAs is also a "mocarhagin protein" as used herein.

[0171] The nucleotide sequence of the clone NMM-2 cDNA is reported as SEQ ID NO:7. Clone NMM-2 was deposited with the Americal Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209589. The protein sequence encoded by clone NMM-2 is reported as SEQ ID NO:8. Amino acids 1-23 of SEQ ID NO:8 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

[0172] The nucleotide sequence of the clone NMM-9 cDNA is reported as SEQ ID NO:9. Clone NMM-9 was deposited with the Americal Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209586. The protein sequence encoded by clone NMM-2 is reported as SEQ ID NO:10. Amino acids 1-23 of SEQ ID NO:10 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

[0173] The nucleotide sequence of the clone NMM-12 cDNA is reported as SEQ ID NO:11. Clone NMM-12 was deposited with the Americal Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209585. The protein sequence encoded by clone NMM-12 is reported as SEQ ID NO:12. Amino acids 1-23 of SEQ ID NO:12 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

[0174] The nucleotide sequence of the clone NMM-13 cDNA is reported as SEQ ID NO:13. Clone NMM-13 was deposited with the Americal Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209584. The protein sequence encoded by clone NMM-13 is reported as SEQ ID NO:14. Amino acids 1-23 of SEQ ID NO:14 are a predicted signal peptide. The mocarhagin propeptide begins with amino acid 24, with the mature protein beginning at amino acid 192.

[0175] Two additional partial cDNAs encoding other closely related proteases (clones "NMM-3" and "NMM-10") were also isolated from the cobra venom gland library as described in Example 5 below. Each of the proteins ecnoded by such cDNAs is also a "mocarhagin protein" as used herein.

[0176] The nucleotide sequence of the clone NMM-3 cDNA is reported as SEQ ID NO:15. Clone NMM-3 was deposited with the Americal Type Culture Collection on Jan. 16, 1998 at accession number ATCC 209587. The protein sequence encoded by clone NMM-3 is reported as SEQ ID NO:16. Amino acids 1-61 of SEQ ID NO:16 are part of the propeptide sequence. The mature mocarhagin propeptide begins with amino acid 62.

[0177] Applicants have also discovered that removal of the mocarhagin propeptide increases the catalytic activity of the enzyme. Thus engineered recombinant forms of mocarhagin include forms having endopeptidase cleavage sites between the propeptide segment and mature peptide segment, including but not limited to, enterokinase cleavage sites or PACE cleavage sites. Alternatively, a propeptide or secretory signal peptide may be substituted for the native mocarhagin propeptide to enable the secretion of active recombinant mocarhagin from eucaryotic host cells.

[0178] The NMM-9 cDNA was used to make a modified construct which includes an enterokinase cleavage sight. Certain preferred embodiments of the present invention included such an enterokinase cleavage site in order to increase production of active (i.e., properly cleaved to remove the leader sequence) protein. The nucleotide

bility may arise by virtue of the presence of a suitable glycosylating enzyme within the host cell, whether naturally occurring, induced by chemical mutagenesis, or through transfection of the host cell with a suitable expression plasmid containing a polynucleotide encoding the glycosylating enzyme. Host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, or HaK cells.

[0195] The mocarhagin protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, Calif., U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No.* 1555 (1987), incorporated herein by reference.

[0196] Alternatively, it may be possible to produce the mocarhagin protein in lower eukaryotes such as yeast, fungi or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* Kluyveromyces strains, Candida, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of expressing heterologous proteins. Suitable fungi strains include Aspergillus sp. or any fungi strain capable of expressing heterologous proteins.

[0197] The mocarhagin protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide encoding the mocarhagin protein.

[0198] The mocarhagin protein of the invention may be prepared by culturing transformed host cells under culture conditions necessary to express a mocarhagin protein of the present invention. The resulting expressed protein may then be purified from culture medium or cell extracts as described in the examples below.

[0199] Alternatively, the mocarhagin protein of the invention is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the mocarhagin protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography.

[0200] Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the mocarhagin protein. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The mocarhagin protein thus purified is substantially free of other mammalian or other host cell proteins and is defined in accordance with the present invention as "isolated mocarhagin protein".

EXAMPLES

[0201] The following examples are presented to illustrate, not to limit, the present invention.

Example 1

~~[0202]~~ Purification of Mocarhagin

[0203] 20 grams of crude protein from snake venom (Naja mossambica mossambica, Sigma, product no. V1627) were dissolved in 500 mL deionized H_2O and centrifuged at 10 K rpm for thirty minutes at 4 C. The supernatant was loaded onto a 200 mL Heparin -650 M affinity column (Toyopearl, Tosohaas) equilibrated with 50 mm Tris-HCl ph 7.6 (buffer A) and 0.2M NaCl. The column was first washed extensively (to baseline) and mocarhagin was eluted with a gradient of 0.2-1.0 M NaCl in buffer A. Fractions containing the protease as monitored by SDS-PAGE (band with molecular weight ~55 kD) were pooled, concentrated using bentriprep-10 (Amicon) and applied to 21.5 mm IDx60 CM size exclusion column (G 3000SW, Tosohaas) in PBS at RT. Fractions eluted from the size exclusion column were analyzed by SDS-PAGE (**FIG. 1**), which showed the presence of multiple proteins of similar molecular weight.

[0204] Fractions containing mocarhagin were pooled and applied onto a Mono S 10/10 column (Pharmacia) equilibrated in 50 mm HEPES ph 8.0 (buffer B) at RT a O-IM NaCl in buffer B, gradient was used to elute the protein. The fractions were assayed by SDS-PAGE, pooled and frozen at 80 C. The recovery was 2-3 mg of mocarahagin per gram snake venom processed with a purity greater than 95%. **FIG. 2** depicts a gel demonstrating the purity of the mocarhagin produced as herein described.

[0205] The N-terminal sequence was determined for the process described above as TNTPEQDRYLQAKKYIEFYVVVDNVMYRKYTGKLHVITXXVYEMNALN (SEQ ID NO:2). The residues indicated in caps (TNTPEQDRYLQAKKYIEFYVVVDNVMYRKY, SEQ ID NO:1) were determined to a higher degree of certainty.

Example 2

~~[0206]~~ Neutrophil/HL60 Binding Inhibition Assay

[0207] Neutrophils were isolated from venous blood anticoagulated with heparin (20 units/mL, final concentration) according to the method of Bignold and Ferrante ((1987) J.

Immunol. Meth. 96, 29). The neutrophils were >95% pure as evaluated by flow cytometry and >98% viable by trypan blue exclusion. HL60 cells were cultured in RPMI medium supplemented with 10% fetal calf serum. Immediately before use, cells were washed twice with phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4). Neutrophils and cultured cells were finally resuspended at 2×10^7/mL in RPMI medium supplemented with 1% fetal calf serum. Binding of ^{125}I-labeled P-selectin (Skinner et al.) to neutrophils or HL60 cells was evaluated by incubating ^{125}I-labeled P-selectin (0.5 μg/mL, final concentration) with cells (1×10^7/mL, final concentration) at 22° C. in a final volume of 200 μl. After 30 min, duplicate 50 μl aliquots were withdrawn and loaded onto 200 μl of 17% (w/v) sucrose in RPMI medium containing 1% fetal calf serum. Neutrophils were pelleted at 8,750× g for 2 min. After careful aspiration of the supernatant, radiolabel associated with the cell pellets was measured in a -counter. Nonspecific binding of ^{125}I-labeled P-selectin was assessed using a 50-fold excess of unlabeled P-selectin (Skinner et al.).

[0208] To examine the effect of pretreatment of neutrophils or HL60 cells with mocarhagin on P-selectin binding, washed cells (2×10^7/mL) in RPMI made 1% in fetal calf serum were incubated in the presence or absence of 10 mM EDTA followed by mocarhagin (0.025-100 μg/mL, final concentrations) for 30 min at 22C. P-selectin binding was then either directly assessed or was assessed after centrifugation of the cells, which were then washed twice and finally resuspended in RPMI with 1% fetal calf serum. In some experiments, DFP-treated mocarhagin was employed in place of mocarhagin. To evaluate the effect of supernatant from mocarhagin treated cells on P-selectin binding, HL60 cells at 10^8/mL in 0.01 M Tris, 0.015 M sodium chloride, 0.001 M calcium chloride, pH 7.4, were incubated with mocarhagin (12 μg/mL) for 10 min at 22° C. The supernatant collected following centrifugation at 1000× g for 10 min was made 0.1% in BSA and loaded onto a heparin Sepharose CL-6B column (0.5×5 cm) to remove mocarhagin. The flow through was then tested for its effect on P-selectin binding to HL60 cells.

Example 3

[0209] PSGL-1 Digestion Assay

[0210] COS cells were cotransfected with three plasmids encoding soluble PSGL-1 (pED.sPSGL-1.T7; Sako et al.), alpha 1,3/1,4 fucosyltransferase (pEA.3/4FT) and soluble PACE (pEA-PACE SOL; Wasley et al. (1993) J. Biol. Chem. 268, 8458-8465). [^{35}S]Methionine-labeled COS conditioned medium containing sPSGL-1.T7 was digested with 5 μg/mL mocarhagin in TBS, 2 mM calcium chloride; 1 mg/mL BSA for 20 min at 37C. The ability of sPSGL-1.T7 to bind P-selectin was assessed by precipitation with the P-selectin IgG chimera LECγ1 (Sako et al.) preabsorbed onto protein A Sepharose beads in TBS, 2 mM calcium chloride, 1 mg/ML BSA for 4 h at 4C. A control experiment was also performed where the LECγ1 protein A Sepharose beads were pre-treated with mocarhagin and then exhaustively washed prior to presentation of sPSGL-1.T7. For immunoprecipitation analysis of untreated and mocarhagin treated sPSGL-1.T7, the protease was inactivated by the addition of 5 mM EDTA. sPSGL-1.T7 was then immunoprecipitated with anti-PSGL-1 polyclonal antibodies Rb3026 (raised against COS produced sPSGL-1.T7; Sako et al.) or Rb3443 (raised against the N-terminal peptide of PACE cleaved PSGL-1:QATEYEYLDYDFLPE, SEQ ID NO:4).

Example 4

[0211] Peptide Cleavage Assay

[0212] A digestion buffer (10 mM MOPS, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, pH 7.5) and a peptide substrate solution (pyroEATEYEYLDYDFLPE (SEQ ID NO:3), 10 mM in DMSO) were prepared. 2.5 mL peptide substrate solution (250 μM final substrate concentration) was combined with mocarhagin sample material (10 μg/L final mocarhagin concentration) and adjusted to 100 μL with digestion buffer using no less than 75 μL. This mixture was digested at 37C. for 16 hours in parallel with a control sample (no mocarhagin added).

[0213] 50 μL aliquots of the digested samples were run on an RP-HPLC column (Vydac C18 218TP54, 4.6×250 mm), using the following solvents: solvent A, 0.1% TFA in H_2O; solvent B, 0.075% TFA in 90% AcN; flow rate 1 mL/min. The presence of peptides in the eluate was measured by absorbance at 214 nm, 260 nm and 280 nm. A positive assay result was indicated by observing elution of two peptide peaks in the tested sample which both elute earlier than the single peptide peak observed in the negative control.

Example 5

[0214] Cloning of Polynucleotide Encoding Mocarhagin Protein

[0215] Venom glands from five Mozambiquan spitting cobras, Naja mossambica mossambica, were dissected at two hour intervals, two to twelve hours following stimulation of venom production. Poly A+RNA was isolated from total RNA of the pooled gland tissue using an Oligotex Direct mRNA kit (Qiagen, Chatsworth, Calif.). Synthesis of cDNA was performed using Superscript Choice System (Gibco BRL, Gaithersberg, Md.) using oligo dT and random hexamer primers, EcoRI adapters. The cDNA was ligated with EcoRI digested lambda Zap II cloning vector (Stratagene, La Jolla, Calif.).

[0216] Using the above cDNA preparation as template, a PCR reaction was performed using degenerate oligonucleotides based on the N-terminal 30 residue amino acid sequence described above. The sequences of the forward primer consisted of 5'-ACNCCNGARCARGAY (SEQ ID NO:19). The sequences of the reverse primer consisted of 5'-RTAYTTYCKRTACAT (SEQ ID NO:20). A resulting 84 bp product was subsequently identified and DNA sequencing confirmed the sequence encoded 30 amino acid residues having a high degree of homology to the previously determined amino acid sequence. Two oligonucleotides 24 nucleotides in length, 5'-CAGGACAGGTACTTGCAGGCCAAA (SEQ ID NO:21) and 5'-ATCGAGTTTTACGTGGTTGTGGAC (SEQ ID NO:22), were synthesized based on the PCR product sequence and used as ^{32}P hybridization probes to screen approximately 10^6 plaques of plated lambda Zap II library. Duplicate sets of Duralose filters (Stratagene, La Jolla, Calif.) were hybridized seperately with each ^{32}P hybridization probe in 5× SSC, 5× Denhardt's, 0.1% SDS, 50 μg/ml yeast tRNA 16 hrs @40C. Filters were washed with 4× SSC,

0.1% SDS @ room temperature, then twice at 45C. for 30 min. Autoradiography was -70C. overnight with intensifying screen. Plaques showing positive hybridization to both probes were isolated and ultimately characterized by nucleotide sequencing.

[0217] Clones NMM-1, NMM-2, NMM-3, NMM-9, NMM-10, NMM-12 and NMM-13, described above, were isolated by this technique.

Example 6

[0218] Enterokinase Cleavage of NMM-9ek

[0219] COS cells were transfected with plasmid pED.NMM9ek, which included the cDNA sequence of SEQ ID NO:17 as an insert. This construct contains a novel enterokinase cleavage site between the propeptide and mature peptide of mocarhagin. After 48hours, the transfected cells were washed in serum free medium, labelled with ^{35}S methionine for 6 hours, and the serum free conditioned mediuma was harvested. Purified bovine enterokinase (La Vallie et al., 1993, J. BIOL. Chem. 268:23311-23317) was added at various concentrations to 100 ul conditioned medium with 10 mM Tris pH 8 and 1 mM $CaCl_2$, and incubate at 37C. overnight. Soy Trypsin Inhibitor resin was added to remove the enterokinase from the reaction mixture. The resin was pelleted by centrifugation and the supernatant was then immunoprecipitated with rabbit polyclonal antibodies raised against mocarhagin purified from cobra venom.

[0220] Following SDS-PAGE and autoradiography, a novel ~50 kD band appeared in the smple lane where 50 nanograms of purified bovine enterokinase had been incubated with the conditioned medium (see **FIG. 3**). This band is consistent with the expected molecular weight of the mature protease when the propeptide (~23 kD) is cleaved off.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 22

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Thr Asn Thr Pro Glu Gln Asp Arg Tyr Leu Gln Ala Lys Lys Tyr Ile
1               5                   10                  15

Glu Phe Tyr Val Val Val Asp Asn Val Met Tyr Arg Lys Tyr
            20                  25                  30
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Thr Asn Thr Pro Glu Gln Asp Arg Tyr Leu Gln Ala Lys Lys Tyr Ile
1               5                   10                  15

Glu Phe Tyr Val Val Val Asp Asn Val Met Tyr Arg Lys Tyr Thr Gly
            20                  25                  30

Lys Leu His Val Ile Thr Xaa Xaa Val Tyr Glu Met Asn Ala Leu Asn
        35                  40                  45
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